Identification and proteomic profiling of exosomes in human urine

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Urine provides an alternative to blood plasma as a potential source of disease biomarkers. One urinary biomarker already exploited in clinical studies is aquaporin-2. However, it remains a mystery how aquaporin-2 (an integral membrane protein) and other apical transporters are delivered to the urine. Here we address the hypothesis that these proteins reach the urine through the secretion of exosomes [membrane vesicles that originate as internal vesicles of multivesicular bodies (MVBs)]. Low-density urinary membrane vesicles from normal human subjects were isolated by differential centrifugation. ImmunoGold electron microscopy using antibodies directed to cytoplasmic or anticytoplasmic epitopes revealed that the vesicles are oriented "cytoplasmic-side inward," consistent with the unique orientation of exosomes. The vesicles were small (<100 nm), consistent with studies of MVBs and exosomes from other tissues. Proteomic analysis of urinary vesicles through nanospray liquid chromatography–tandem mass spectrometry identified numerous protein components of MVBs and of the endosomal pathway in general. Full liquid chromatography–tandem MS analysis revealed 295 proteins, including multiple protein products of genes already known to be responsible for renal and systemic diseases, including autosomal dominant polycystic kidney disease, Gitelman syndrome, Bartter syndrome, autosomal recessive syndrome of osteopetrosis with renal tubular acidosis, and familial renal hypomagnesemia. The results indicate that exosome isolation may provide an efficient first step in biomarker discovery in urine.

A major goal in the field of clinical proteomics is to identify disease biomarkers in biological fluids that can be measured relatively inexpensively for early diagnosis of disease. An important challenge in this process is to develop a rational means of reducing the complexity of the proteome of body-fluid samples to enhance the detectability of relatively low-abundance proteins that may have special pathophysiological significance. Most of the focus thus far has been on proteomics of blood serum or plasma (1). Because urine can be collected noninvasively in large amounts, it provides an attractive alternative to blood plasma as a potential source of disease biomarkers (2).

The water channel aquaporin-2 (AQP2) is one biomarker that can be readily measured in urine (3) and that has been exploited in studies of various water-balance disorders (4). AQP2 is an integral membrane protein, and investigators thus far have been puzzled with regard to the mechanism of its secretion into the urine. Biochemical studies (5) and immunoelectron microscopy (6) have demonstrated that AQP2 is present in small, low-density membrane vesicles but have not provided an explanation for the appearance of these vesicles in the urine. Immunoblotting of urinary membrane fractions has revealed that, in addition to AQP2, the kidneys excrete membranes containing apical plasma-membrane transporter proteins from each renal tubule segment (5), suggesting that analysis of urinary membrane fractions could provide noninvasive information about the pathophysiological state of the entire renal tubule.

We hypothesize that AQP2 and other apical plasma-membrane proteins are excreted through the process of exosome formation, i.e., delivery of the internal vesicles of multivesicular bodies (MVBs) to the urinary space by fusion of the outer membrane of MVBs with the apical plasma membrane of renal tubule epithelial cells. This hypothesis leads to explicit predictions that can be tested directly. First, the urinary vesicles should be oriented "cytoplasmic-side inward," a unique characteristic of the internal vesicles of MVBs (7). Second, the urinary vesicles should be small (<100 nm in diameter) and relatively uniform in size, consistent with the internal vesicles of MVBs (8) and exosomes secreted by other tissues (7). Third, the urinary vesicles should contain proteins typical of MVBs and of exosomes formed by other cell types (7).

Here we use immunoelectron microscopy and nanospray liquid chromatography–tandem MS (LC–MS/MS) analysis of urinary membrane proteins to show that AQP2 and other apical plasma-membrane proteins are excreted through the process of exosome formation in agreement with the predictions above. The proteomic analysis revealed the presence of 21 proteins known to be associated with specific renal diseases or blood pressure regulation. These studies demonstrate the potential for using the urinary exosomes as a starting material in studies aimed at disease biomarker discovery.

Methods

A full description of the methods is provided in *Supporting Methods*, which is published as supporting information on the PNAS web site. A brief synopsis follows.

Subjects, Sample Collection, and Sample Processing. Six normal male volunteers (aged 28–74, Approved Clinical Research Study no. 04-H-0025) urinated directly into sterile 50-ml plastic centrifuge tubes containing 4.2 ml of a protease inhibitor mixture. The samples were mixed together and centrifuged at 17,000 × *g* for 15 min at 4°C to remove whole cells, large membrane fragments, and other debris. The supernatant was centrifuged at 200,000 × *g* for 1 h at 4°C to obtain a low-density membrane pellet. Kidney samples for immunoblotting were obtained from the unaffected portion of a kidney resected because of a renal tumor (approved as exempt from review by the National Institutes of Health Office of Human Subjects Research).

Depletion of Tamm–Horsfall Protein (THP). In one of the collected urine samples, THP was removed by incubating the resuspended low-density membrane pellet with the reducing agent DTT and repeating the ultracentrifugation. This procedure denatures the zona pellucida domains in the THP, thus inhibiting aggregation and allowing THP to be removed in the supernatant.

Immunoblotting and Antibodies. Immunoblotting was carried out as described in ref. 9. Briefly, low-density membrane pellets were solubilized in Laemmli sample buffer to a final concentration of 1.5% SDS, 6% glycerol, and 10 mM Tris·HCl (pH 6.8). Proteins

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Abbreviations: APN, aminopeptidase N; AQP1 and AQP2, aquaporin-1 and -2; LC–MS/MS, liquid chromatography–tandem MS; MVBs, multivesicular bodies; NCC, thiazide-sensitive Na–Cl cotransporter; THP, Tamm–Horsfall protein; VPS, vacuolar protein-sorting.

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were separated by 1D SDS/PAGE and were transferred to nitrocellulose membranes, which were blocked and probed with antigen-specific primary antibodies (described in *Supporting Methods*). Blots were incubated with species-specific horseradish peroxidase secondary antibodies, followed by a luminol-based chemiluminescence reagent, and then were exposed to light-sensitive film.

Immunoelectron Microscopy. Vesicle suspensions were mixed 1:1 with 4% paraformaldehyde and then were applied to 200-mesh nickel grids. After blocking with 1% BSA and washing, the grid was incubated with primary antibody containing 0.02% Triton X-100 (to permeabilize the vesicle membranes) for 45 min at room temperature. Grids were exposed to primary antibodies recognizing AQP-2, thiazide-sensitive Na–Cl cotransporter (NCC), aminopeptidase N (APN), or CD9 and then were exposed to species-specific anti-IgG antibodies conjugated to colloidal gold particles (6 nm or 12 nm) (Jackson ImmunoResearch). After washing, membranes underwent negative staining with 0.5% uranyl acetate. After drying, the grids were examined with a JEOL 1200 EX electron microscope operated at 60 kV. Control labeling was performed identically, but nonimmune IgG was substituted for the primary antibody.

Initial Separation and In-Gel Trypsin Digestion of Proteins. Low density membranes were prepared and solubilized as described above, with (experiment 1) or without (experiment 2) depletion of THP. Proteins (experiment 1, 40 μg; experiment 2, 300 μg) were separated by 1D SDS/PAGE with 10% polyacrylamide. Gels were stained with colloidal Coomassie blue. After destaining, the gel was cut with a razor blade into small blocks from top to bottom (experiment 1, 54 blocks; experiment 2, 37 blocks). Proteins in the gel blocks were reduced, alkylated with iodoacetamide, trypsinized, and extracted as described in ref. 10. The trypsin digestion was performed by using 12.5 ng/μl Sequencing Grade Modified Trypsin (Promega) diluted in 25 mM NH_4HCO_3 solution. The extracted peptides were dried and reconstituted with 0.1% formic acid before analysis by nanospray LC–MS/MS.

Nanospray LC–MS/MS. Tryptic peptides from each gel block were analyzed by 1D nanospray LC–MS/MS with a modified ProteomeX 2D LC/MS workstation (Thermo Finnigan, San Jose, CA). Chromatographic separation of peptides was accomplished by using two Zorbax 300SB-C18 peptide traps (Agilent Technologies, Wilmington, DE) in alternating fashion (replacing the standard strong-cation-exchange and reverse-phase columns); the standard electrospray ionization source was replaced by a nanospray ionization source and a reversed-phase PicoFrit column [BioBasic C18, 75 μm $\times$ 10 cm, tip = 15 μm (New Objective, Woburn, MA)]. The peptides were loaded onto the traps in alternating fashion by using a Surveyor autosampler (Thermo Electron, San Jose, CA). After washing with 0.1% formic acid, the peptides were eluted by 0–60% solvent B in solvent A (A = 0.1% formic acid, B = acetonitrile) in 30 min at a flow rate of about 200 nl/min (75 μl/min before splitting).

Raw data files were searched against the human protein sequence database by using BIOWORKS software (Thermo Finnigan). In addition to the standard measures of analysis provided by the software, all collision-induced dissociation (CID) spectra were manually inspected for quality. In addition, the protein identification was further validated manually through BLAST (National Center for Biotechnology Information) and HARVESTER searches (European Molecular Biology Laboratory, Heidelberg, Germany; http://harvester.embl.de).

Results

Orientation of Urinary Vesicles. If low-density urinary vesicles isolated from human urine are exosomes, as hypothesized, they should have a unique orientation, with the cytoplasmic side inward, because of their mechanism of formation by invagination of the outer membrane of MVBs (7). This contrasts with endosomes, which are oriented cytoplasmic-side out. ImmunoGold electron microscopy (Fig. 1) of gently permeabilized (0.02% Triton X-100) vesicles was carried out with antibodies to epitopes on the cytoplasmic side of the membrane (AQP2 and NCC) and on the external (anticytoplasmic) side [APN and CD9 (a tetraspanin)]. With APN and CD9, labeling was largely outside of the membrane bilayer. With AQP2 and NCC, labeling was consistent with an internal localization of these epitopes. Therefore, the orientation of these vesicles is consistent with the hypothesis that they are exosomes. Furthermore, the labeling provided further evidence that these vesicles derive from epithelia throughout the renal tubule [APN, proximal tubule (11); CD9, thick ascending limb (12); NCC, distal convoluted tubule (13); AQP2, collecting duct (14)].

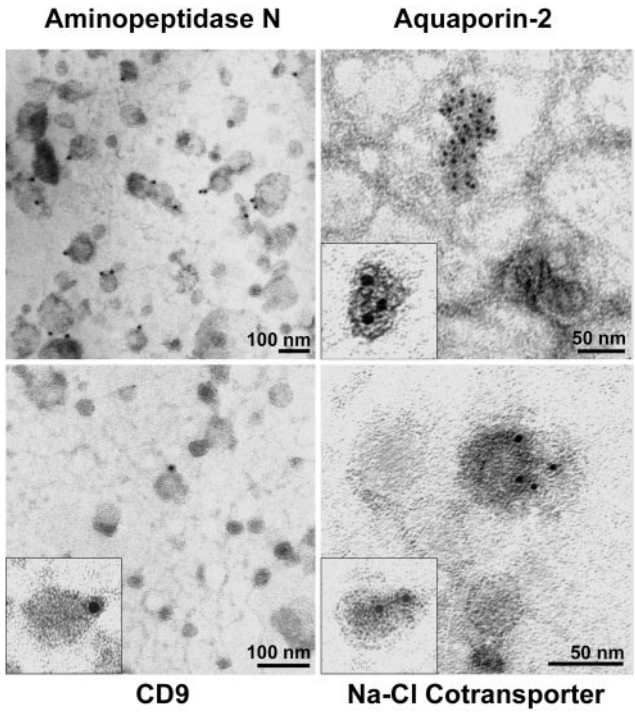

Fig. 1. Immunoelectron microscopy of urinary vesicles. ImmunoGold labeling using antibodies to membrane proteins targeted to external epitopes (APN and CD9) or cytoplasmic epitopes (AQP2 and NCC). *Insets* show selected fields at increased magnification.

Size Distribution of Urinary Vesicles. Quantitative analysis of electron micrographs of negatively stained urinary vesicles revealed that these vesicles are relatively small. A typical electron micrograph of negatively stained urinary vesicles (Fig. 2*A*) illustrates that the vesicle diameters are frequently only 5- to 10-fold greater than the bilayer thickness, typical of the internal vesicles of MVBs. Analysis of the distribution of vesicle size (Fig. 2*B*) shows a mode of 35–40 nm, consistent with the small size of exosomes from other tissues (7).

Proteomic Analysis of Urinary Vesicles. For proteomic analysis of the urinary vesicles, 1D SDS/PAGE was carried out and the gels were sliced into consecutive blocks from top to bottom (Fig. 3*A*). These gel blocks were subjected to in-gel trypsinization, and the extracted peptides were analyzed by nanospray LC-MS/MS. An immunoblot (Fig. 3*B*) showed that the abundant protein centered at $\approx$90 kDa was THP, an apically expressed, glycosylphosphatidylinositol-linked protein ex-

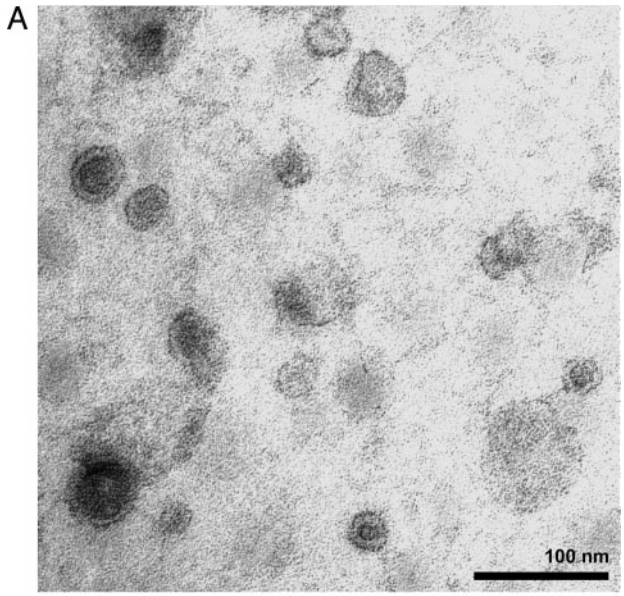

Fig. 2. Size distribution of urinary vesicles. (*A*) Electron micrograph of negatively stained urinary vesicles. (*B*) Histogram of vesicle diameter.

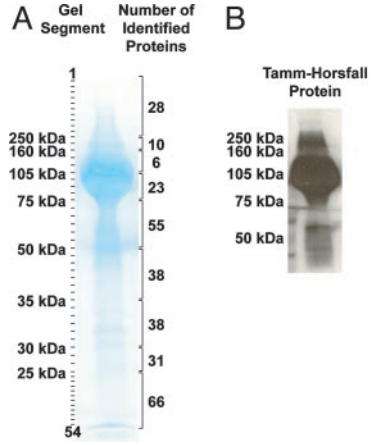

Fig. 3. 1D gel electrophoresis of urinary vesicles. (*A*) Coomassie-blue-stained gel of urinary vesicles. Shown is one of the two gels analyzed in the present study. The number of proteins identified in each molecular mass range is indicated. (*B*) Immunoblot of the same sample with anti-THP antibody; the large bulge is due to a large amount of THP.

pressed in the thick ascending limb of Henle. THP was also readily identifiable by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS when proteins were separated by 2D electrophoresis (Fig. 8, which is published as supporting information on the PNAS web site). A second 1D gel was analyzed (data not shown) after removal of THP from the final pellet by reduction with DTT and reultracentrifugation to increase the identification of low-abundance proteins in the region of gel previously occupied by THP.

Overall, from the two gels, 295 unique proteins were identified (Fig. 4 and Table 3, which is published as supporting information on the PNAS web site). This included 73 proteins (24.7% of total) known to be involved in membrane trafficking, chiefly in the endosomal pathway. The LC-MS/MS analysis identified multiple small GTP-binding proteins, including proteins in the Rab, ARF, Rho, and Ral families (Table 3). In addition, there were 10 cytoskeletal and motor proteins. There were 48 integral membrane proteins (putative cargo proteins), including several transporters and channels associated chiefly with the apical plasma membrane. Furthermore, there were eight glycosylphosphatidylinositol-linked proteins (expressed in apical plasma membranes) and 23 peripheral-membrane proteins. In contrast, there were few proteins identified that are expressed predominantly in the nucleus, mitochondria, endoplasmic reticulum, or Golgi apparatus (Fig. 4). The cytosolic proteins that were identified were presumably trapped in the lumina of forming exosomes. Interestingly, although there were a large number of proteins identified that are known to be expressed in apical plasma membranes, no integral membrane proteins were identified that are known to be expressed exclusively in the basolateral plasma membrane (Table 3). We identified ubiquitin throughout the molecular weight range investigated, suggesting that ubiquitinated proteins were present in most fractions (Fig. 5).

Several class E vacuolar protein-sorting (VPS) proteins (associated with MVB biogenesis) were identified (Table 1). Furthermore, the list of proteins identified in this study includes 50 proteins previously identified in exosomes derived from other cell types (Table 4, which is published as supporting information on the PNAS web site), further supporting the conclusion that the low-density urinary membranes isolated from urine by ultracentrifugation consist largely of exosomes.

Immunoblotting was carried out to confirm the presence of selected proteins in the low-density membrane fraction used for the proteomic analysis (Fig. 6). In this manner, we confirmed the presence of 19 proteins previously identified by MS and also identified the three subunits of the amiloride-sensitive sodium channel family (not seen with MS). Many of the proteins found

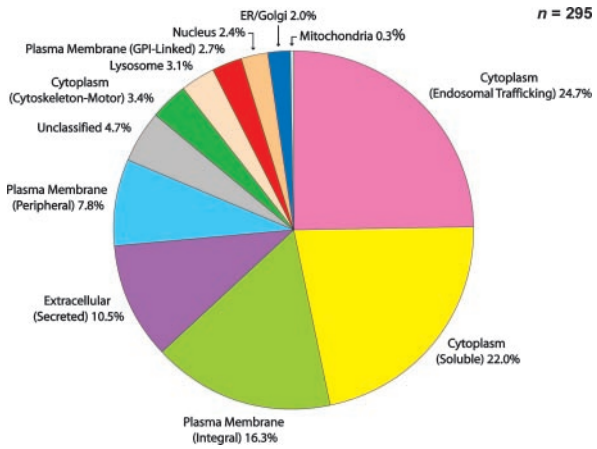

Fig. 4. Distribution of identified proteins by subcellular origin. Protein classification is based on analysis by using a HARVESTER search. Protein classes are color-coded to correspond to Table 3.

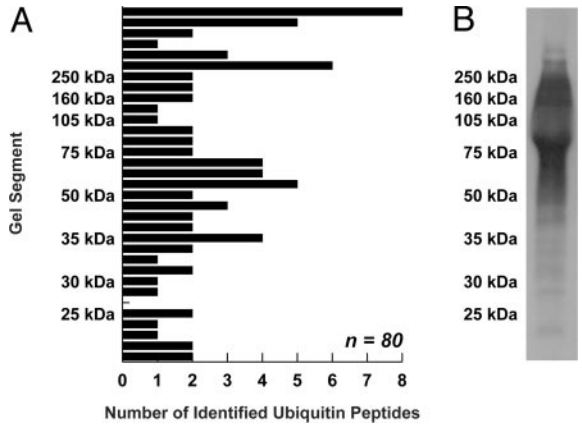

Fig. 5. Instances of identification of ubiquitin. Ubiquitin was identified by LC–MS/MS (*A*) and immunoblot by using anti-ubiquitin antibody (*B*) on 1D gels over a molecular mass range from ≈10 kDa to ≈400 kDa, presumably representing a variety of ubiquitinated proteins.

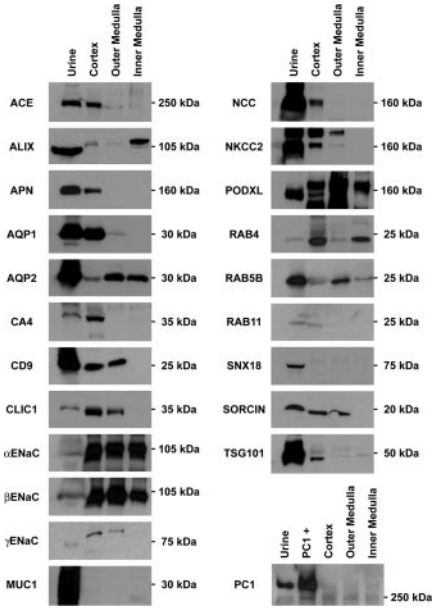

Fig. 6. Immunoblots of proteins in human urine exosomes (Urine) and kidney regions (Cortex, Outer Medulla, and Inner Medulla). Immunoblots were probed with antibodies to the following proteins: ACE, angiotensin-converting enzyme; ALIX, ALG-2 interacting protein X; APN; AQP1; AQP2; CA4, carbonic anhydrase type IV; CD9; CLIC1, chloride intracellular channel-1; αENaC, epithelial sodium channel α; βENaC, epithelial sodium channel β; γENaC, epithelial sodium channel γ; MUC1, mucin-1; NCC; NKCC2, Na–K–2Cl cotransporter type 2; PODXL, podocalyxin; RAB4; RAB5B; RAB11; SNX18, sorting nexin-18; sorcin; TSG101; and PC1, polycystin-1. In the PC1 immunoblot, an additional lane was added (PC1+), which was loaded with PC1 protein extracted from Madin–Darby canine kidney cells with stable expression of human PC1 protein (a kind gift of Feng Qian, The Johns Hopkins University, Baltimore).

in the low-density urinary membrane fraction (i.e., exosomes) were markedly enriched relative to kidney homogenates from cortex, outer medulla, and inner medulla.

Several of the putative cargo proteins identified are associated with recognized forms of renal or systemic diseases, including hypertension (Table 2). One important example is polycystin-1, the protein product of the gene responsible for autosomal dominant polycystic kidney disease, the most common genetic disease leading to renal failure. Polycystin-1 is of low abundance in kidney tissue but is readily detectable in urinary exosomes.

Discussion

Analysis of urinary excretion of AQP2 has proven very useful in clinical studies of polyuric states (4). With the premise that other disease biomarker proteins may be identifiable in urine, we have carried out a proteomic analysis of a low-density membrane fraction isolated from urine.

To interpret changes in the excretion of membrane proteins, it is important to understand the mechanism of the excretion process. Sasaki and his colleagues (3) proposed that the lipidic membranes in urine are derived from intracellular vesicles that are somehow released into the urine. Here we have provided evidence supporting the view that the intracellular vesicles are exosomes, that is, the internal vesicles of MVBs that are excreted into the urine by fusion of the MVB outer membrane with the apical plasma membrane of renal epithelial cells.

Three types of evidence support this conclusion. First, the urinary vesicles were found to have a unique cytoplasmic-side inward orientation that is characteristic of the internal vesicles of MVBs but is opposite to what would be expected for either secretory vesicles or endosomes (7). Second, the size distribution of the excreted vesicles matches that seen in exosomes from other sources and conforms to the size criterion for exosomes proposed by Thery and colleagues (7). Many of the exosomes found were extremely small. As shown in Fig. 2*A*, many were only 5–10 times the thickness of the limiting membrane bilayer, which is 3–4 nm (33). Vesicles from other sources, such as sloughing of

Table 1. Identified class E VPS proteins involved in MVB targeting and biogenesis

Complex/domain	Protein name	GI no.	Yeast	Refs.
ESCRT-I	VPS28 isoform 1	7705885	VPS28p	15–17
	TSG101	5454140	VPS23p/STP22p	15–17
UEV	Signaling molecule ATTP*	23943814	VPS23p/STP22p	18
ESCRT-III	CHMP1.5	41150865	DID2p/CHM1p	19–23
	CHMP2/BC-2	7656922	VPS2p/DID4p	19–23
	CHMP2.5/CGI-84	40254866	VPS2p/DID4p	19–23
	CHMP4B/SHAX1	28827795	VPS32p/SNF7p	19–23
	CHMP5/CGI-34	6841574	VPS60p/MOS10p	19–23
BRO1	ALIX	22027538	VPS31p/NPI3p/BRO1p	24
BRO1-like	Hypothetical protein FLJ32421*	21389601	VPS31p/NPI3p/BRO1p	25
AAA ATPase	VPS4A	7019569	VPS4p	26
	VPS4B	17865802	VPS4p	26

*Proteins potentially involved in MVB biogenesis. Signaling molecule ATTP is a partial paralogue of human TSG101. Hypothetical protein FLJ32421 contains BRO1-like domain.

Table 2. Proteins identified in urinary vesicles that are associated with kidney diseases or hypertension

Protein name	GI no.	Kidney diseases or hypertension
AQP2	4502179	Autosomal recessive nephrogenic diabetes insipidus, type 1 [MIM:222000] Autosomal dominant nephrogenic diabetes insipidus, type 1 [MIM:125800]
Sodium potassium chloride cotransporter-2	4557849	Antenatal Bartter syndrome type 1 [MIM:601678]
NCC	4506977	Gitelman syndrome [MIM:263800]
Epithelial sodium channel α	4506815	Autosomal recessive pseudohypoaldosteronism type 1 [MIM:264350] Liddle syndrome [MIM:177200]
Epithelial sodium channel β	4506817	
Epithelial sodium channel γ	42476333	
FXYD domain-containing ion transport regulator-2	11125766	Familial renal hypomagnesemia [MIM:154020]
Carbonic anhydrase II	4557395	Autosomal recessive syndrome of osteopetrosis with renal tubular acidosis [MIM:259730]
Carbonic anhydrase IV	4502519	Proximal renal tubular acidosis [MIM:114760]
Polycystin-1	33359213	Autosomal dominant polycystic kidney disease type 1 [MIM:173900]
Uromodulin	4507833	Medullary cystic kidney disease 2 [MIM:603860] Hyperuricemic nephropathy, familial juvenile [MIM:162000]
Podocin	7657615	Autosomal recessive steroid-resistant nephrotic syndrome [MIM:600995]
Nonmuscle myosin heavy chain IIA	12667788	Fechtner syndrome [MIM:153640] Epstein syndrome [MIM:153650]
Adenine phosphoribosyltransferase	4502171	2,8-Dihydroxyadenine urolithiasis [MIM:102600]
Angiotensin I converting enzyme isoform-1	4503273	Hypertension [MIM:106180]
Aminopeptidase A	36951116	Hypertension (27)
APN	4502095	Hypertension (28)
Aminopeptidase P	10880126	Hypertension (29)
Neprilysin	4505203	Hypertension (30)
Hydroxyprostaglandin dehydrogenase 15 (NAD)	31542939	Hypertension (31)
Dimethylarginine dimethylaminohydrolase-1	6912328	Hypertension (32)

MIM, Mendelian Inheritance in Man database no.

apical plasma membrane components or apoptosis, would be expected to be bigger and more varied in size (34). Third, the roster of proteins identified strongly supports the view that the urinary vesicles are derived from MVBs (Fig. 7). In particular, many so-called class E VPS proteins were identified. Yeast orthologs of these proteins have been found to mediate MVB formation (Table 1). The identified proteins include members of the ESCRT-I protein complex (involved in recognition of ubiquitinated cargo by MVBs), the ESCRT-III protein complex (involved in invagination of the MVB outer membrane), ALIX (an ESCRT-III binding partner), and VPS4 (an AAA ATPase that interacts with ESCRT complexes). In addition, the LC–MS/MS analysis of the urinary vesicles identified 50 proteins that have previously been identified in exosomes from other cell types, such as B lymphocytes and dendritic cells (Table 4). All together, the analysis identified 73 proteins recognized to be involved in membrane trafficking processes (Fig. 4 and Table 3). In addition, there were several cytoskeletal proteins and putative endosomal cargo, including apical plasma membrane proteins and cytosolic proteins. In contrast, the analysis identified few proteins associated with other organelles, such as mitochondria or nucleus, and no proteins that are exclusively targeted to the basolateral plasma membrane. Thus, we conclude that low-density urinary vesicles that can be isolated from urine by differential centrifugation are largely made up of exosomes derived from the apical plasma membrane endocytic pathway.

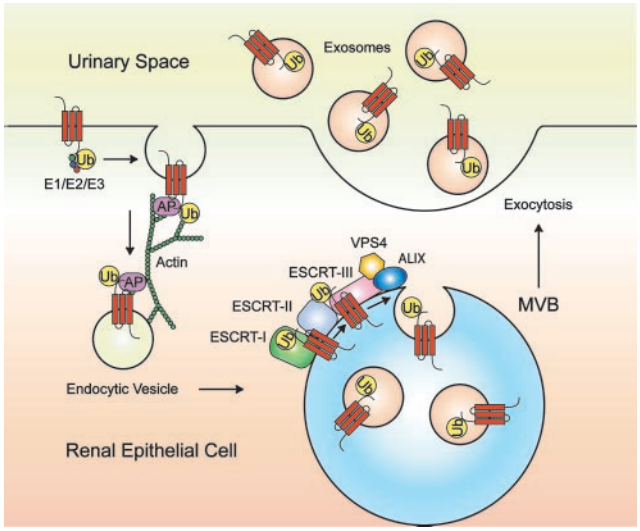

Fig. 7. Process of exosome formation and release into the urine. Ub, ubiquitin; AP, adaptor protein; ESCRT, endosomal sorting complex required for transport; ALIX, ALG-2 interacting protein X.

The process of MBV formation and exosome secretion into urine is summarized in Fig. 7. The signal that marks plasma-membrane proteins for incorporation into MVBs is *monoubiquitination* (15). This signal is distinct from *polyubiquitination*, which targets proteins to the proteasome for degradation. The LC–MS/MS analysis demonstrated E2 and E3 components of the ubiquitination apparatus, as well as ubiquitin itself. Interestingly, ubiquitin was identified throughout the molecular weight range of the analysis (Fig. 5), suggesting the presence of many ubiquitinated proteins in the urinary exosomes, although the data shown do not distinguish between mono- and polyubiquitinated proteins. Ubiquitinated proteins are endocytosed and sorted in the endosomal pathway by a process dependent on adaptor proteins that couple ubiquitinated proteins to the clathrin coats in clathrin-mediated vesicle budding (35), followed by vesicle transport via the actin cytoskeleton. The ubiquitinated cargoes are recognized by ESCRT-I and then segregated in the MVB outer membranes and internalized by membrane invagination through actions of ESCRT-II, ESCRT-III, VPS4, and ALIX. In this model, the outer membrane of MVB fuses with the apical plasma membrane, releasing internal vesicles, exosomes,

to the urinary space. The orientation of exosomes is different from that of endosomes, which are oriented with their outside surfaces facing the cytosol. When the endosomes fuse with the outer membrane of MVBs and then invaginate to form intra-MVB vesicles, they turn inside out, with cytosol trapped inside of the vesicles. Therefore, exosome secretion into the urine delivers discrete packets of cytosol from renal epithelia to the urine, providing a potential means for noninvasive detection and analysis of protein-expression changes in renal tubule cells.

The LC–MS/MS analysis of exosomes identified proteins from renal epithelia extending from the glomerular podocytes (podocin and podocalyxin) through the proximal tubule (e.g., megalin, cubilin, APN, AQP1, type IV carbonic anhydrase, and γ-glutamyltransferase), the thick ascending limb of Henle (e.g., THP, CD9, and the type 2 Na–K–2Cl cotransporter), the distal convoluted tubule (e.g., NCC), and the collecting duct (e.g., AQP2, mucin-1, and the Rh type C glycoprotein). Furthermore, highly abundant proteins of the transitional epithelium of the urinary bladder, uroplakin-1 and -2, were identified. Thus, proteomic analysis of urine can potentially provide insight into the physiological or pathophysiological processes in every epithelial cell type facing the urinary space.

Although no attempt was made in this study to investigate abnormalities in the urinary proteome in specific patient populations, our analysis of the urinary exosome proteome of normal human subjects identified several proteins known to be involved in specific diseases of the kidney or of blood pressure regulation (Table 2). One example is carbonic anhydrase type II. As early as 1990, Sato *et al.* (36) demonstrated that urinary membranes of normal subjects contain both carbonic anhydrase II and carbonic anhydrase IV (as confirmed here) but that there was a complete lack of immunoreactive carbonic anhydrase II in urinary membranes from patients with one form of autosomal recessive metabolic acidosis. Also, of particular interest is the ready detectability of polycystin-1 in the exosome fraction of urine, which was confirmed by immunoblotting (Fig. 6). Mutations in the gene that codes for this protein account for a common genetic disease, type 1 autosomal dominant polycystic kidney disease, one of the most common monogenetic diseases and a prevalent cause of end-stage renal disease. It remains for future studies of well characterized patient populations to determine whether proteomic analysis of urinary exosomes can provide a tool for early detection of renal disease. Furthermore, it has recently been recognized that exosomes exist in serum (37), providing a potential approach to discovery of disease biomarkers in blood samples.

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